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Research Article

Recombinant scorpine: a multifunctional antimicrobial peptide with activity against different pathogens

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Abstract. Scorpine is an antimicrobial peptide whose structure resembles a hybrid between a defensin and a cecropin. It exhibits antibacterial activity and inhibits the sporogonic development of parasites responsible for murine malaria. In this communication we report the production of scorpine in a heterelogous system, using a specific vector containing its cloned gene. The recombinantly expressed scorpine (RScp) in *Anopheles gambie* cells showed antibacterial activity against *Bacillus subtilis* and *Klebsiella pneumoniae*, at 5 and 10 μM, respectively. It also produced 98 % mortality in sexual stages of *Plasmodium berghei* at 15 μM and 100 % reduction in *Plasmodium falciparum* parasitemia at 5 μM. RScp also inhibited virus dengue-2 replication in C6/36 mosquito cells. In addition, we generated viable and fertile transgenic *Drosophila* that overexpresses and correctly secretes RScp into the insect hemolymph, suggesting that the generation of transgenic mosquitoes resistant to different pathogens may be viable.

Keywords. Antimicrobial peptide, dengue, malaria, *Plasmodium*, recombinant scorpine, virus.

Introduction

A wide variety of living organisms produce a large repertoire of antimicrobial peptides (AMPs) that play an important role in innate immunity to microbial invasion [1–5]. AMPs exhibit rapid killing, often within minutes *in vitro,* and a broad spectrum of activity against Gram-positive and Gram-negative bacteria, fungi, parasites, enveloped viruses and tumor cells [2, 4, 6–8]. It is generally accepted that positively charged peptides interact directly with the negatively charged cellular membranes of bacterial cells, resulting in increase of membrane permeability, which leads to a rapid cell death [1, 2, 9], although intracellular targets after membrane permeabilization have also

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been proposed [2, 9–13]. For example, buforin II enters and accumulates in the bacterial cytoplasm, interfering with cellular function by binding to DNA and RNA [14]. Attacins block the synthesis of integral membrane proteins [15], and PR-39 inhibits DNA synthesis [16]. Pyrrhocoricin, an insect peptide, kills bacteria by binding DnaK [13], thus preventing protein-refolding function and leading to bacterial death.

AMPs also have potent activity against malaria parasites. Magainins [17], defensins [18] and cecropins expressed by mosquitoes disrupt sporogonic development of *Plasmodium* parasites [19, 20]. Similarly, synthetic AMPs have been shown to interfere with the development of *Plasmodium* [21–23]. Together these results have established the basis for a strategy aimed at producing transgenic mosquitoes resistant to malaria by heterologous expression of AMPs that could interrupt malaria parasite transmission.

Several cationic amphipathic peptides also display antiviral activity *in vitro.* Defensins neutralize papillomavirus [24], herpes simplex virus (HSV) [25–27], vesicular stomatitis virus, influenza virus [3, 28] and HIV [29–34]. Tachyplesins [35] and polyphemusins [36] are active against vesicular stomatitis virus, influenza A virus and HIV [32]. Melittins, cecropins [33, 34] and indolicidin [32] display anti-HIV activity. Peptide T22, derived from polyphemusin II, inactivates HIV-1 *in vitro* by binding to both the virus gp120 protein and the T-helper cell CD4 receptor, blocking virus-cell fusion in an early stage of the infection [37]. Melittin and cecropins inhibit the replication of HIV-1 by suppressing the long terminal repeat gene [34]. Recently, it was proposed that the theta defensin retrocyclin 2 inhibits influenza virus infection by cross-linking and immobilizing surface glycoproteins, resulting in blockage of viral entrance into the cell [38]. However, the mechanism of action of most AMPs against parasites and viruses remains unclear. The production of recombinant AMP facilitates functional and structural studies. For example, recombinant human cathelicidin-derived LL-37 has facilitated the resolution of its three-dimensional structure using nuclear magnetic resonance (NMR) spectroscopy.

Many antimicrobial peptides have been described in vertebrates and invertebrates such as lepidopterans [39]; less work has been done with arachnids. The discovery of antimicrobial peptides in scorpion venom brought new insights into the mode of action of venom and also opened new avenues for the discovery of novel antibiotic molecules from arthropod sources. Consensus of the role of AMPs peptides in scorpion venom peptides has yet to be reached, although several functions have been postulated: (1) AMPs may protect the scorpion from bacterial infection via the pair of ducts that directly connect the venomous glands to the external environment; (2) AMPs may depolarize neural cells inducing immobilization of prey, allowing the scorpion to capture it [40]; (3) and the AMPs could potentiate the action of other neurotoxins found in the venom.

Scorpions are a rich source of antimicrobial peptides: (1) androctonin isolated from the hemolymph of *Androctonus australis*, which contains two disulfide bridges and shows marked sequence similarity to tachyplesins, polyphemusins and gomesin [41, 42]; (2) buthinin, which is a three-disulfide-bridge bactericidal and fungicidal peptide also isolated from the hemolymph of the same scorpion [41]; (3) hadrurin from the venom of *Hadrurus aztecus,* which is hemolytic [43]; (4) parabutoporin from the venom of the South African scorpion *Parabuthus schlechteri* [44]; (5) opistoporin-1, which possesses hemolytic activity, and opistoporin-2, both from the venom of the South African scorpion *Opistophtalmus carinatus* [45]; (6) pandinin-1 and -2, two alpha-helical polycationic peptides [46]; and (7) scorpine [47], which is the subject of this communication, arising from the venom of *Pandinus imperator.*

Scorpine was shown to have anti-bacterial and antiplasmodial activity *in vitro* [47]. In this communication, we have extended our functional studies using recombinant scorpine (RScp). It has shown a potent toxic effect on sexual and asexual stages of *Plasmodium berghei* and *P. falciparum,* respectively, and also a strong inhibition of dengue 2 virus (DENV-2) infection. These data make RScp a very attractive molecule for the development of transgenic mosquitoes that could eventually interrupt the transmission of dengue fever and/or malaria.

Materials and methods

Plasmid construction

In order to place the scorpine gene under control of the *Anopheles gambiae* serpin promoter, the N-serpin10tTA-N plasmid [48] was digested with *Eco*RI and *Bam*HI, and the 700-bp fragment corresponding to the serpin promoter was inserted into the plasmid pKSEscp [47]. The serpin-scorpine cassette (1.2-kbp *Sal*I/*Eco*RI fragment) was then inserted into a derivative of the TETOP plasmid [48] to add the BgH terminator 3′ to the scorpine coding sequence. Finally, the resulting plasmid, N-SEscp-BgH, was digested with *Not*I to release the scorpine expression cassette, which was then inserted into the *Minos* transposon, pMinHygeGFP<NotI> [49]. The integrity of the resultant plasmid (MinSEscp) (Fig. 1) was confirmed by diagnostic restriction enzyme digestion

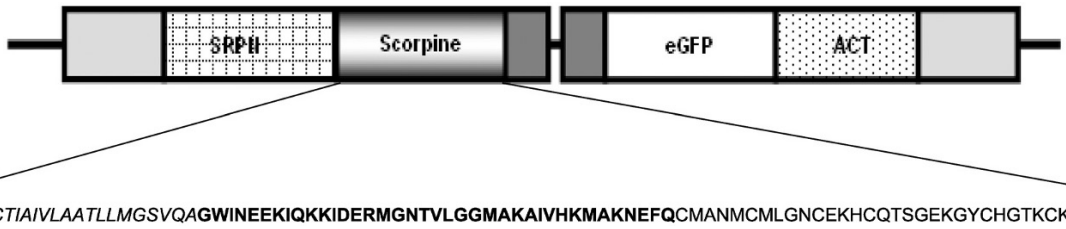

Figure 1. Schematic representation of plasmid MinSEscp used for mosquito cell transfection. The light gray boxes represent *Minos* ends. *Minos* sequence was interrupted by insertion of the scorpine gene [in the N-terminus the signal peptide sequence is shown in italics (dark gray box), the cecropine-like sequence is in bold, and the further sequence corresponds to the defensin-like sequence] under the control of serpin promoter (SRPN), and the enhanced version of the green fluorescent protein (eGFP) under the control of the actin promoter (ACT). Dark gray boxes represent the BgH terminator sequence.

and was shown to contain the scorpine expression cassette, together with the eGFP gene under the control of *Drosophila* actin 5C promoter, flanked by the right and left terminal repeats of the *Minos* transposable element.

Cell culture and transfections

A. gambiae cell line Sua 5.1* (kindly provided by H. Müller, EMBL, Heidelberg) was maintained in Schneider medium supplemented with 10% FCS. The *Aedes albopictus* cell line C6/36 (kindly provided by F. Medina-Ramírez, CINVESTAV, Mexico) was maintained in MEM medium supplemented with 10% FCS and 1% vitamin (Gibco). Baby hamster kidney cells, BHK-21, were grown in MEM medium supplemented with 10% FCS (Gibco) at 37°C/5% CO_2 (kindly provided by J. Ruíz-Gómez, CISEI-INSP, Mexico).

Sua 5.1* cells were transfected using the Effectene transfection Reagent (Qiagen) according to the manufacturer's instructions and incubated for 48 h at 28°C to allow RScp expression. The total amount of DNA used was 2 μg of MinSEscp plasmid. Cell transfection efficiency was determined by fluorescent flow cytometry by measuring eGFP expression.

Immunolocalization of RScp in transfected cells

Transfected and non-transfected cells grown on multiwell plates (Nunc) were rinsed with PBS, fixed in FEM solution (3% paraformaldehide, 1 mM EGTA, 2 mM $MgCl_2$) for 10 min and washed in PBS. The cells were incubated with a rabbit polyclonal antibody against native scorpine (1:500) for 1 h at room temperature (RT). After washing (4 × 5 min PBS), rhodamine conjugated goat anti-rabbit antibody (Pierce, 1:500 dilution in 3% BSA/PBS) was added for 30 min and then washed 4 × 5 min PBS. Cells expressing RScp were detected by fluorescence microscopy (Nikon, Eclipse E-600).

Recombinant scorpine quantification

Seventy-two hours post-transfection, supernatant from MinSEscp transfected Sua 5.1* cells was concentrated by centrifugation into Centricon® columns (30 kDa molecular cutoff, Millipore) at 7500 x *g* for 1 h. RScp was quantified by Western slot blot densitometry using purified native scorpine as a reference. Scorpine was purified in the laboratory following the technique earlier described [34]. Groups of four slots were filled either with 100 μl of concentrated supernatant or 0.03, 0.06, 0.13 and 0.250 μg of native scorpine in quadruplicate, whereas the unused slots were filled with 100 μl of PBS and filtered. The membrane was incubated with a rabbit polyclonal antibody against native scorpine (1:5000 dilution in 1% non-fat milk, 0.1% Triton X-100 in PBS) for 2 h at room temperature. The membrane was washed three times (PBS/0.1% Triton X-100) and incubated at room temperature with anti-rabbit peroxidase-linked secondary antibody (1:40000) (Amersham) for 1 h. After washing, the blots were developed using Super Signal Chemiluminescent-HRP (Pierce), and the RScp concentration was estimated from the native scorpine standard curve.

Recombinant scorpine immunoblot analysis

The presence of RScp in supernatant of transfected cells was analyzed by Western blot. The equivalent of 1 μg of RScp was analyzed by electrophoresis on 18% polyacrylamide/SDS gel. The electrotransfer to a nitrocellulose membrane (Amersham) and detection of RScp were carried out as described above.

Scorpine antibody production

The antibody against scorpine was generated in a rabbit (adult New Zealand). For this purpose highly purified native scorpine was injected subcutaneously in the back of the animal at four different times. The first injection contained 50 μg of pure scorpine, plus 175 μl of water and 225 μl of complete Freund's adjuvant. This was repeated 4 weeks later, using incomplete Freund's adjuvant. The third injection,

applied 4 weeks later, contained 100 μg of pure scorpine, plus 125 μl of water and 225 μl of incomplete Freund Adjuvant. A serum sample was taken from the rabbit 2 weeks after the third inoculation, and the titer of antibodies against scorpine was evaluated (1 : 5 000) by an ELISA, using pure scorpine attached to the plates. A final injection using the same conditions as the second one was performed at completion of 6 months. After 2 weeks, the entire serum was collected. This material was used for identification of scorpine.

Anti-bacterial assays

The method of Fleming et al. [50] was used to assay the anti-bacterial activity of RScp. *Bacillus subtilis* and *Klebsiella pneumoniae* inoculums (200 μl of a 0.1 A_{600} culture containing 3.2×10^8 colony-forming units cfu/ml), from the CISEI/INSP bacteria collection, were plated onto LB agar plates. The surface of the medium was allowed to dry for about 3 min. Sterile paper disks (13 mm diameter) were impregnated with 1 and 2 μM RScp for *B. subtilis* and 6 and 10 μM for *K. pneumoniae*. Disks were soaked in the appropriate RScp solution for 3 min. The excess liquids were drained by gently patting with filter paper and were placed onto the agar surface plated with bacteria. Inhibition zones were recorded around wells in thin agar plates with bacteria, as described by Hoffman et al. [51]. Non-transfected cell supernatant was used as negative control.

Parasites

The gametocyte-producing *Plasmodium berghei* Anka strain 2.34 (kindly provided by R. S. Sinden, Imperial College of Science, Technology and Medicine, UK) was used. Parasites were maintained in BALB/c mice by mechanical passage. Mice with parasitemias ranging between 50 and 60% and gametocytemia around 10% (counted in Giemsa-stained blood smears) were bled by heart puncture using heparin (100 IU/ml blood).

Plasmodium falciparum FCR3 strain was cultured in human erythrocytes type A+ at 5% hematocrit. Parasites were cultured in RPMI (Gibco) medium supplemented with HEPES 25 mM, glutamine 2 mM, glucose 2 g/l, $NaHCO_3$ 2 g/l, hypoxanthine 29.25 mg/l, gentamicin 60 mg/l and Albumax 1.6% at pH 7.4 [39]. Cultures were kept in 96% nitrogen, 3% CO_2 and 1% oxygen atmosphere at 37°C, and fresh medium was added every 24 h [52].

Effect of RScp on *P. berghei* ookinete development and *P. falciparum* asexual stages

In two independent experiments, *P. berghei*-infected mouse blood was resuspended 1:5 in RPMI culture medium, and 500 μl aliquots were placed into individual wells of flat-bottom 24-well plates and incubated for 24 h at 21 °C to allow ookinete development. RScp from transfected cell supernatant at a final concentration of 15 μM was added at different times (0 min, 15 min, 30 min, 3 h, 24 h) during the culture to the wells containing infected blood in duplicates. After 24-h culture, the number of ookinetes/10 000 erythrocytes was determined in Giemsa-stained blood smears. Cultured human erythrocytes at 2.5% of parasitemia (*P. falciparum* asexual stages) were cultured in the presence of increasing concentrations of RScp (0.1 – 10 μM) for 16, 40, 64 and 88 h. Every 24 h the medium was discharged, and fresh medium with the appropriate RScp concentration was added. The percentage of infected red blood cells was determined by microscopic examination of thin blood films stained with Giemsa. Each RScp concentration was tested by duplicate. Controls with RScp solvent (Schneider culture medium) and non-treated infected erythrocytes were included.

Effect of RScp on dengue-2 virus infection

Confluent monolayers of BHK-21 cells in 96-well plates were cooled to 4 °C and infected with 0.1 PFU of DENV-2 NGC strain from the CISEI/INSP (Mexico) dengue virus collection. This strain had the following passage history: three consecutive times through suckling mouse brain, followed by three successive passages onto C6/C36 cell line and finally once more in suckling mouse brain. The viral stock was titrated by plaque assay. Cells were kept with dengue virus for 1 h at 4°C. Unbound virus was removed by washing the cells three times with a cool medium. Fresh medium containing 0.25 and 0.5 μM of RScp was added to cells and kept for 4 days at 37 °C/5% CO_2 and observed by bright field microscopy. Cell viability was determined by Trypan blue exclusion.

Dengue negative strand determination

C6/36 cells infected with 1.0 PFU of DENV-2 NGC strain (as described above) were collected at 12 and 24 h for RNA extraction. RNA (2 μg) was converted to cDNA with the GeneAmp® RNA PCR kit (Applied Biosystems) with sense primer Den 2_oligo 5(F): 5′ GCAGAACCTCCATTCGGAGACAGCTACAT 3′ according to the manufacturer's instructions. cDNA was subjected to 40 PCR amplification cycles with sense and antisense primers Den 2_oligo 5(F): 5′ and Den 2_oligo 6(R): 5′ AGCTCACAACGCAACCACTAT 3′. Each cycle consisted of cDNA denaturation for 30 s at 94 °C, annealing of primers for 30 s at 55 °C, and primer extension for 30 s at 72 °C. The resulting fragment of 393 bp for the negative strand was determined by electrophoresis in agarose gels.

Dengue immunoblot analysis

Mock infected and infected C6/36 cells with 1.0 PFU of DENV-2 NGC strain were collected at 48 h post-infection; the cells were lysed with 100 µl of RIPA buffer (Tris-HCl 50 mM pH 7.4, NP-40 1%, Na-deoxycholate 0.25%, NaCl 150 mM) with a protease inhibitor cocktail (EDTA 1 mM, PMSF 1 mM, aprotinin, leupeptin, pepstatin 1 µg/ml each) by gentle pipetting, and the lysates were incubated in ice for 15 min. After centrifugation (12 000 × *g* 15 min), supernatant was collected and subsequently resolved in a 10% SDS-PAGE gel and transferred onto a Hybond C membrane. The membrane was blocked with 5% non-fat milk, 0.1% Triton X-100 in PBS overnight, washed with 0.1% Triton X-100 in PBS (washing buffer) and incubated with mouse anti-NS3 antibody (non-structural protein 3) (kindly provided by R. Padmanabhan, Georgetown University, Washington, DC) 1 : 1000 dilution in 1% non-fat milk, 0.1% Triton X-100 in PBS for 2 h at room temperature. The membrane was washed three times with washing buffer and incubated at room temperature with an anti-rabbit peroxidase conjugated antibody (1 : 10 000) for 1 h and washed three times. The NS3 band was visualized by the Super Signal Chemiluminescent-HRP substrate system (Pierce).

***Drosophila* strains and transgenic fly constructions**

Previous studies demonstrated that *Drosophila* is an adequate model to assay bacterial and parasite infections [53]. It is for this reason that *Drosophila* was used as a standardized model insect system for *in vivo* expression of scorpine [54].

Drosophila Oregon R and yw strains were used as control organisms. Scorpine cDNA was cloned in pCasperhsp83, and the transgenic flies were constructed following a reported protocol [55].

To construct a hybrid molecule between scorpine and a signal peptide, a consensus signal peptide sequence was designed from different defensin sequences reported in the GenBank. The sequence chosen is shown in the 'Results' section. This DNA was made by recursive PCR and cloned in the 5′ end of a partial cDNA encoding the complete mature scorpine peptide, essentially as described earlier by our group [54]. Since in the previous publication no details of the nucleotides used were reported, in the present report the full sequence is provided (see 'Results').

The frame was verified by DNA sequence and then cloned in the *Drosophila* expression vector Casperhsp83. To verify the RNA expression of the scorpine lines, total RNA from control and transgenic flies was purified from adult organisms. After cDNA synthesis, typical RT-PCR was performed using specific primers for the scorpine RNA. The PCR products were analyzed in agarose gels.

Transgenic lines were balanced using the double balancer line *w/w; SP/CyO; MKRS/TM3*. Fertility and viability of the transgenic organisms was determined by calculating the number of adult flies of the expected class after crosses of heterozygous balanced transgenes.

RScp identification in transgenic flies

To identify the presence of mature scorpine peptide in the *Drosophila* hemolymph, adult flies were anaesthetized and centrifuged in spin columns with PBS as buffer, and the filtrated reaction was run in SDS-gel electrophoresis. Identification of RScp was performed by Western blot experiments using the specific anti-scorpine polyclonal antibody prepared in our laboratory.

Results

RScp production

Anopheles gambiae cells (Sua 5.1*) were transfected with MinSEScp plasmid (Fig. 1) to express RScp (Fig. 2A). Supernatants of Sua 5.1* transfected cells analyzed by Western blot revealed the presence of a 10-kDa protein that was recognized by the polyclonal scorpine antiserum (Fig. 2B).

The expression construct includes the consensus signal peptide from defensins (2 kDa), which together with the processed peptide encodes a 10 kDa protein. Apparently the extra peptide added at the N-terminal sequence of scorpine was not eliminated from the expressed scorpine. The product recovered from the Sua 5.1* cells might still contain this extra segment of peptide, judging by the molecular weight of the expressed product. However, since the vector contains the correct nucleotide sequence that codes for recombinant scorpine and the product is recognized by antibodies generated against the native peptide, this was taken as clear evidence that scorpine or a hybrid containing the scorpine sequence is present and active. Furthermore, RScp from supernatant of transfected mosquito cells when assayed for anti-microbial activity against two bacterial species, two plasmodial species (*P. berghei* and *P. falciparum*) and a DENV-2 line was shown to express the expected activity (anti-bacterial, inhibitory action on *Plasmodium* development and, additionally, anti-viral activity).

RScp anti-bacterial activity

It was very important to demonstrate that Rscp was capable of producing inhibition of bacteria growth

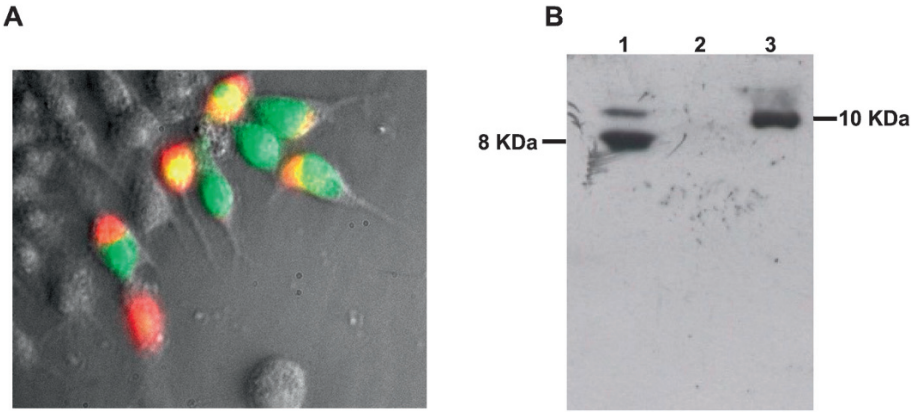

Figure 2. RScp production in transfected *Anopheles gambiae* cells. (*A*) RScp localization in transfected Sua 5.1* cells. GFP expression (green), RScp localization (red). (*B*) Western blot assay of transfected and non-tranfected mosquito cells is shown: purified native scorpine from the African scorpion venom (1), supernatant from non-transfected (2) and transfected (3) Sua 5.1* cells. The additional band seen in lane 1 run with native scorpine is possibly a dimer of the peptide.

Table 1. Effect of RScp on *P. falciparum* trophozoite development.

RScp concentration (μM)	Infected red blood cells (%)				
	0 h	16 h	40 h	64 h	88 h
10	2.5	0.4 ± 0.2	0	0	0
7	2.5	0.6 ± 0.2	0	0	0
5	2.5	0.7 ± 0.2	0	0	0
2	2.5	1.0 ± 0.2	0.8 ± 0.2	0.4 ± 0.2	0
0.7	2.5	1.2 ± 0.3	1.0 ± 0.3	0.6 ± 0.3	0
0.5	2.5	1.2 ± 0.2	1.2 ± 0.1	0.7 ± 0.1	0
0.3	2.5	1.1 ± 0.2	1.2 ± 0.2	0.8 ± 0.2	0
0.1	2.5	1.5 ± 0.1	1.1 ± 0.1	0.8 ± 0.1	0
Solvent	2.5	3.6 ± 0.4	4.8 ± 0.2	6.0 ± 0.3	12 ± 2
Negative control	2.5	4.0 ± 0.2	5.0 ± 0.4	5.4 ± 0.6	14 ± 1

Recombinant scorpine was added at different concentrations (10, 7, 5, 2, 0.7, 0.5, 0.3 and 0.1 μM) to *P. falciparum* cultures. The number of infected erythrocytes was counted as described in the 'Materials and methods' section.

similar to that originally shown for native scorpine as purified from *P. imperator* venom [47]. It is for this reason that in this communication the two different species of bacteria earlier used by Conde et al. [47] were assayed by the method described by Fleming et al. [50]. The minimal concentrations able to inhibit the growth of *B. subtilis* and *K. pneumoniae* were 1.0 and 10 μM, which correspond to inhibition zones of bacterial growth of 2.4 and 1.5 mm, respectively.

RScp anti-plasmodial activity

RScp (15 μM final concentration) was added to *P. berghei*-infected mouse blood at different times during culture to observe potential effects on the development of ookinete stages. Reduced numbers of ookinetes were observed in all experimental wells in comparison to untreated cultures, but the highest inhibition of ookinete formation (98%) occurred when RScp was added at time 0, when gamete formation and fertilization occur (Fig. 3). These results are similar to those obtained with native scorpine [47]. The dose response was determined using *P. falciparum* trophozoite stage cultures that were exposed to RScp. All concentrations tested (0.1–10 μM) reduced parasite density over the time course of the experiment in relation to controls. At 16 hours post-treatment, the maximum inhibitory effect was observed at the highest concentration (10 μM) of RScp (Table 1). But, a critical dependence on exposure time was also observed in RScp treated cultures. At 40 h of exposure, no parasites were observed at RScp 5 μM or above (Table 1). In addition, no infected erythrocytes were detected after 88 h, at all concentrations tested (Table 1).

RScp anti-viral activity

Many AMP's have antiviral activity and it was particularly interesting to determine whether DENV-2 replication and viral-induced cytopathy were modified by exposure to RScp. Initially, the effects of RScp were analyzed in susceptible BHK-21 cells, since mosquito cell lines do not show clear cellular morphology changes following DENV-2 infection [56]. BHK-21 infected cells with DENV-2 in

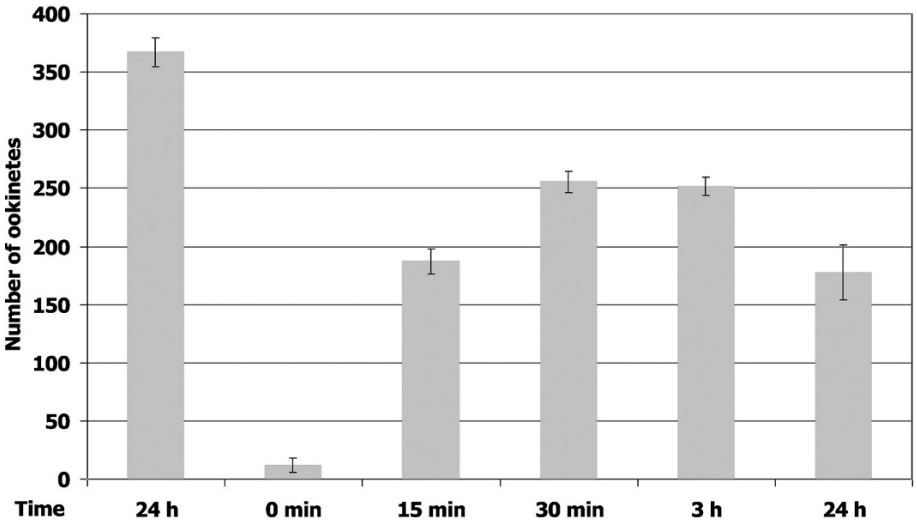

Figure 3. Effect of RScp on *Plasmodium berghei* ookinete development. *P. berghei* sexual stages were cultured for 24 h. RScp was added at different times (0 min, 15 min, 30 min, 3 h and 24 h) during the culture. The number of ookinetes was assessed at 24 h as described in the 'Materials and methods' section. Negative controls were untreated cultures.

the presence of RScp 0.5 μM showed no changes in cellular morphology (Fig. 4A). However, a clear cytopathic effect (clump formation) was noted at the lower concentration tested (0.025 μM), which suggests a dose response between concentration and antiviral activity (Fig. 4B). No changes in morphology were observed in non-treated cells (Fig. 4C) compared with BHK-21 infected cells (Fig. 4D), where a clear cytophathic effect was observed.

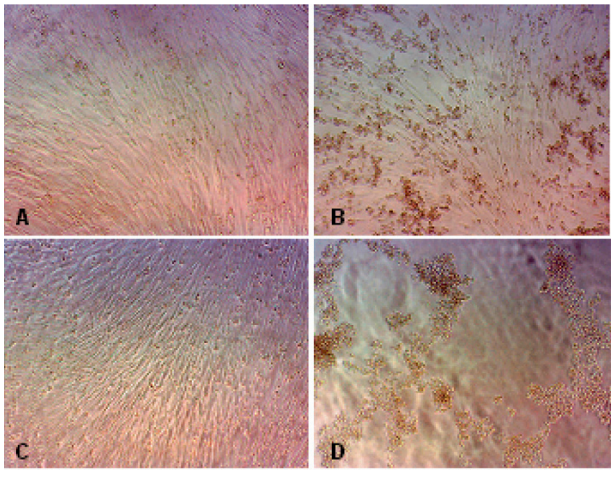

Figure 4. Inhibition of dengue cell infection by RScp. BHK-21 cells infected with DENV-2 were treated with 0.5 (*A*) and 0.025 μM (*B*) of RScp, mock infected cells (*C*) and cells infected with dengue virus in the absence of RScp (*D*) were used as controls. The cytophatic effect was observed by phase contrast microscopy.

Since mosquito cells allow DENV-2 viral replication (although no obvious cellular effects of viral infection are observed), we examined the synthesis of negative-strand viral RNA in mosquito cells exposed to RScp at concentrations known to inhibit viral replication in BHK-21 cells. RT-PCR was conducted to detect nsRNA on C6/36 cells infected with DENV-2 and exposed to 0.5 μM RScp.

DENV-2 nsRNA (negative RNA strand) was produced and accumulated during the 24-h experiment (compare line 2, 12 h, and line 2,24 h, Fig. 5). When cells were treated with RScp, it was possible to detect nsRNA (line 3, 12 h, Fig. 5).

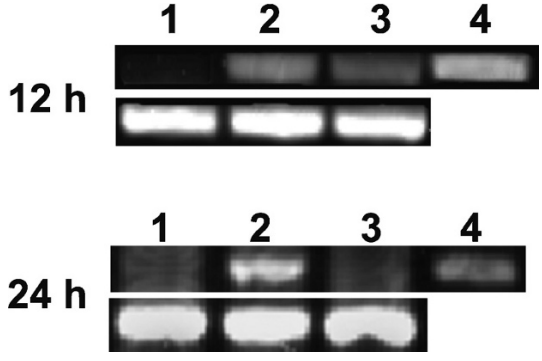

Figure 5. Inhibition of DENV replication by RScp. Cells were incubated with RScp and dengue replication was analyzed at 12 and 24 h by RT-PCR. Non-infected C6/36 cells (1), C6/36 cells infected with DENV-2 (2), treated with 0.5 μM of RScp (3) and plasmid-positive control (4).

The concentration of NS3 was determined by Western blot on cells infected with 1 PFU of virus and treated with or without RScp. Figure 6 shows that NS3 levels on cells treated with RScp were almost undetectable (compare line 3 and line 1) in contrast to non-treated cells.

Expression of RScp in transgenic *Drosophila*

At this point, the results indicated that RScp protein can be expressed and maintain its activity in cultured mosquito cells. However, it was important to know

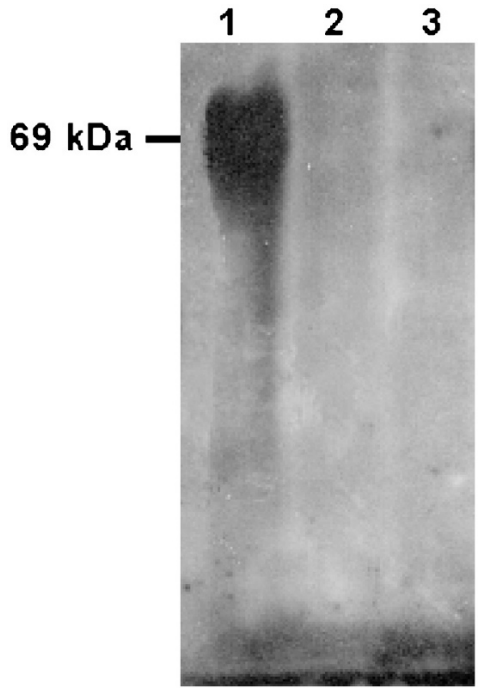

Figure 6. NS3 expression in C6/36 infected cells. (1) Infected cells with DENV-2, (2) non-infected cells and (3) treated cells with 2 μM of RScp were analyzed by Western blot.

whether RScp can be overexpressed in a complete organism (*in vivo* expression). Three independent transgenic *Drosophila* lines (named S1, S2 and S3) were obtained. The scorpine gene expression in adult transgenic flies was investigated by RT-PCR experiments. It was found that only the S1 and S2 lines expressed the scorpine cDNA (Fig. 7B). Since the scorpine peptide was fused to a signal secretory peptide (Fig. 7A), we assumed that the scorpine would be preferentially located in the hemolymph of adult organisms. Hemolymph protein extracts from adult transgenic and the parental yw organisms were obtained and analyzed by Western blot experiments using an anti-scorpine specific antibody prepared for this work. Figure 7C shows that a peptide of the expected molecular weight was present in the hemolymph of transgenic flies, but absent in the parental line. Very little signal was detected in fly carcasses (data not shown), suggesting that most of the scorpine was secreted. The transgenic flies overexpressing scorpine were viable and fertile and did not show any evident defects during the insect development and reproduction, indicating that scorpine overexpression is not toxic to the fly (Table 2). These results suggest that RScp peptide can be expressed and secreted in an insect hemolymph, a mechanism that is required for its eventual expression in mosquitoes.

Discussion

Several antimicrobial peptides have been described from scorpions [41–46, 57] including scorpine, a 75-amino acid peptide isolated from *Pandinus imperator* venom [47]. Native scorpine purified from venom glands has a molecular mass of 8350 Da. It has a peculiar structure compared to other known AMPs. Its N-terminal amino acid sequence is similar to cecropins, whereas its C-terminal region has several disulfide bridges, similar to the structure of defensins [47].

RScp expressed from mosquito cells appears to be 2 kDa greater in size than the native molecule. This would suggest that the consensus signal peptide is not cleaved prior to secretion into the medium and may indicate that RScp is being secreted by the type I secretion system (SSTI). In this system, proteins move directly from the cytoplasm to the extracellular space and are commonly employed for toxin, protease and lipase secretion. There are several examples of secreted proteins whose signal peptides are not cleaved, including fibroblast growth factor (FGF)-9 [58], plasminogen activator inhibitor-2 [59] and the carp retinol-binding protein (RBP) [60]. Alternatively, RScp may have the signal peptide removed but carry other post-translational modifications as a con-

Table 2. Viability rates in transgenic *Drosophila* flies expressing RScp.

Genotype	% of organisms of the expected class that develop into adults[C]
yw/yw;S1/Cyo x yw/yw; S1/SPa	84% (77/92)
yw/yw;S2/TM2 x yw/yw; S2/MKRSb	83% (65/78)
yw x ywd	90% (133/147)

[a] Transgenic RScp line *S1* is located at the second chromosome and was balanced with the *CyO and SP* chromosomes to maintain the transgene insert. However, homozygous *S1* flies were viable and fertile.
[b] Transgenic RScp line *S2* is located at the third chromosome and maintained with the *TM2* and *MKRS* balancer chromosomes. Homozygous *S2* flies were viable and fertile.
[c] The expected class includes heterozygous flies with the balancer chromosome and the transgene chromosome as well as homozygous transgenic flies. A viability effect caused by the balancer chromosome was expected in the heterozygous organisms. The parenthesis indicates the number of organisms observed against the number of expected flies.
[d] Transgenic construct was injected in *yw* embryos; thus *yw* organisms were used as the control-parental strain.

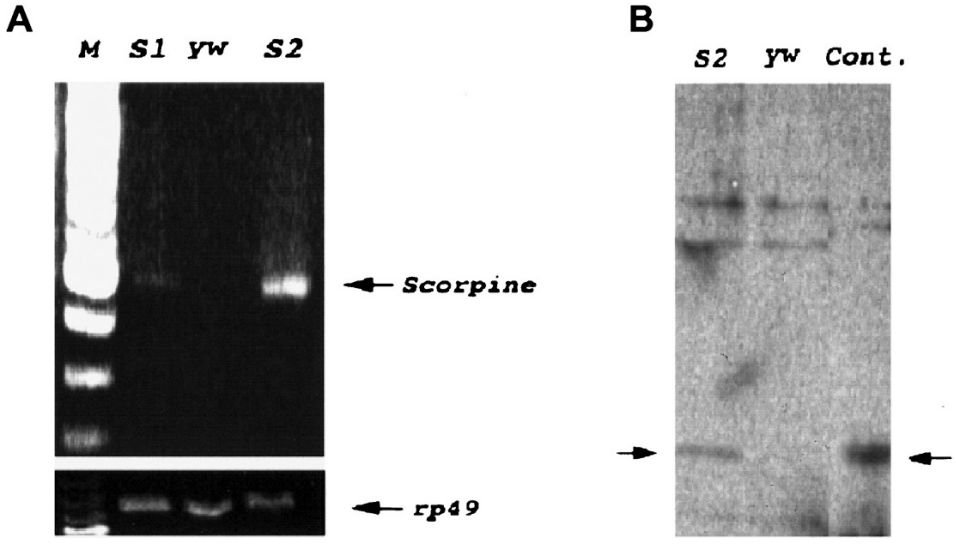

Figure 7. Scorpine expression in transgenic *Drosophila.* (*A*) Ethidium bromide staining RT-PCR products from adult transgenic flies and yw flies as control. S1 and S2 correspond to RT-PCR products of the scorpine transgenic flies, and yw is the reaction from the parental line. M is DNA markers, and rp49 RT-PCR product was used as the internal control. (*B*) Western blot experiment using an anti- scorpine antibody. Total hemolymph protein extracts from S2 transgenic flies (S2) and control flies (yw) are shown. As control, 20 ng of pure scorpine peptide from the African scorpion *P. imperator* was included.

sequence of production in a mosquito cell line, which leads to the observed molecular weight difference. However, as we selected a consensus sequence taken from various peptides in order to generate a signal peptide, it is possible that the sequence added remained as an extra segment expressed at the N-terminal extremity of scorpine. Intriguingly, the RScp expression (containing a signal peptide present in some defensins in transgenic flies) seems to have been correctly processed and secreted in the fly hemolymph, suggesting that the process of RScp secretion in *Drosophila* tissues may be different from that in mosquito cells.

RScp produced in *A. gambiae* cells showed toxicity to bacteria and *P. berghei* gametes and zygotes like that described for native scorpine [47]. We further demonstrated similar toxic effects towards *P. falciparum* asexual stages. In addition, RScp inhibits dengue-2 virus replication and the production of NS3 virus protein. These results demonstrated that RScp maintains its biological activity against bacteria and humans, but also rodent malaria parasites.

Magainins and cecropins have been reported to disrupt sporogonic development of *P. knowlesi, P. gallinaceum and P. cynomolgi* [19, 20]. Synthetic AMPs have been shown to interfere with the development of *Plasmodium.* For example, shiva-3 has *in vitro* toxicity towards the sexual phases of *P. berghei* at a concentration of 100 μM [22, 23]. Similarly, during *in vivo* studies with shiva-3, the prevalence and intensity of *P. berghei* infection was reduced at 50 μM [21]. The only peptide showing more potent antiplasmodial activity is a dermaseptin S4 aminoheptanoyl-derived peptide (19–21 μM). However, this peptide also displays hemolytic activity at active concentrations [61], like the human cathelicidin-derived LL-37 [62] and the magainin analogue MSI-78 [63]. Recently, it was shown that other scorpine-like molecules can be slightly toxic to erythrocytes and to oocytes [64].

In comparison, hemolysis was not observed with RScp (data not shown), and the anti-plasmodial effect is observed at the lowest concentration (5 μM) seen to date. These results suggest that RScp is a good candidate to generate transgenic mosquitoes to block parasite transmission.

The most common mechanism of action for AMPs to kill Gram-negative bacteria and parasites is the 'carpet' model, where positively charged regions of their α-helical peptides bind to negatively charged lipids on the membrane [11, 12, 65–67], causing an increase in surface area that weakens the bilayer. This alteration leads to transient pore formation [68], transport of outer lipids to the inner leaflet and finally collapse of the membrane into small AMP-coated vesicles. An alternative model of activity called the 'barrel-stave' mechanism proposes that α-helical peptides bundle on the surface of the membrane and assume an orientation to allow the hydrophobic surfaces to interact with the lipid core, while the hydrophilic surfaces orient inward [66]. This arrangement creates an aqueous channel. Death of the microbe would then result from either loss of polarization [69], leaking of cellular contents [67], disturbance of membrane function from lipid redistribution

[12] or activation of hydrolases that destroy the cell wall [70]. Recently, the inhibition of intracellular targets was also proposed [15, 71, 72]. However, a mechanistic anti-bacterial and parasiticidal action for RScp awaits identification.

RScp also showed anti-viral activity on DENV-2. DENV-2 is an envelope RNA virus belonging to the *Flavivirus* genus of the *Flaviviridae* family. The main sequential events of dengue viral replication are attachment of the virus particle to an unknown receptor on the plasma membrane; internalization of the virion in non-clathrin vesicles; release of the viral genome to the cytoplasm after pH-dependent fusion of the viral and vesicle membranes; translation of the positive-strand viral genome, synthesis of nsRNA, followed by generation of the progeny genomic RNA strand, and packaging of virus particles by the replicase complex [73]. As indicated above, some AMPs display antiviral activity at several stages on the virus replication process. It is hypothesized that, since nsRNA is produced during mosquito cell infection in the presence of RScp, the attachment, internalization and uncoating of the virion are not affected by RScp. But we observed viral replication inhibition, since two components of the replicase complex were not present on infected cells treated with RScp (Figs. 5 and 6). However, other factors can contribute to the anti-viral activity displayed by RScp. For example, it is accepted that AMPs can alter the structure of biological membranes [74], and it is therefore possible that RScp could disturb endoplasmic reticulum membranes in a way that exponential replication of the virus cannot be achieved, as DENV-2 replication depends on the integrity of the endoplasmic reticulum membranes [75]. Further experiments will help elucidate this point. It is also possible that RScp could inhibit viral polyprotein processing by inhibition of the viral protease NS2B-NS3, similar to the effect of small cationic antimicrobial peptides, such as odorranain B (ORB) [76], although there is no significant similarity between ORB and RScp (not shown). Overall, these experiments show that RScp affects DENV-2 replicase complex.

From the experiments reported herein there are several conclusions: Recombinant AMP expression is highly valuable for structural studies, as shown by recent studies on LL-37, the only human member of the cathelicidin family, a cationic peptide of 37-amino acid residues showing an amphipathic alpha-helical structure [77, 78]. The production of RScp opens the possibility of its structural characterization by circular dichroism (CD) and NMR spectroscopy. This analysis could provide considerable insight into the three-dimensional structure of the peptide and its relation to various aspects of its anti-microbial activities, as well as on how it disrupts and/or translocates across target membranes.

On the other hand, recombinant scorpine has anti-bacterial and anti-viral activities as well as anti-parasitic activity against *Plasmodium*, using both *in vivo* and *in vitro* assays. It can be expressed in cell lines and in intact insect (*Drosophila*), where it does not seem to be toxic for the survival and reproduction of offspring. Thus, the eventual production of a transgenic mosquito carrying and expressing the gene that codes for scorpine or a similar peptide is a project worth serious research effort.

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